

REMARKS

I. Status of the Claims

Claims 14 and 34-47 were pending. Claims 14 and 34-44 are amended. Claims 45-47 are canceled. Claims 48-51 are newly added. No new matter has been added. Claims 14, 34-44 and 48-51 are pending.

In response to the Examiner's response concerning the Election/Restriction set forth in the above-mentioned Office Action, Applicants have cancelled claims withdrawn from consideration. Applicant specifically reserves the right to file one or more divisional applications directed to the non-elected and/or cancelled subject matter.

The amendments to the claims are merely of an editorial nature. Support for the high stringency recitation introduced into claim 14, and commonly known in the art, finds support for example at page 11, lines 5-10. The newly added claims are identical or almost identical to the already examined claims. Thus, no new matter has been added by the present response. For example, claims 48 and 49 are identical to claims 35 and 36, respectively, but dependent on claim 34 instead of claim 14; and claims 50 and 51 are almost identical to claim 34 except for their dependencies.

Reconsideration in view of the following remarks and entry of the foregoing amendments are respectfully requested.

II. Rejections Under 35 U.S.C. §112, First Paragraph

Claims 34, 36-41, 43 and 44 have been rejected under 35 U.S.C. § 112, first paragraph as failing to comply with the written description requirements as lacking enablement.

The Examiner alleges that the "rejection over claims 34, 36-41, 43 and 44 is necessitated" by the previous amendments to the claims. The Examiner objects to "(d)" in claim 14 and

notably to “an amino acid” in “(i)” together with the “having at least 95% identity” recitation in “(ii)”. The Examiner contends that the genus encompassed by claim 14 is not commensurate with the description. Applicant respectfully submits that in view of the present amendment which replaces “an” by “the” in “(i)” and the addition of “and having a sodium ion channel activity” in “(ii)”, that the rejection has been overcome. It is believed that the claimed genus shares a common core structure (the sequence) and/or function (sodium ion channel activity). Applicant also wishes to stress the fact that the present invention is not only enabling for the alpha subunit of the SCN3A sodium channel and mutants thereof, but also for that of two additional alpha subunits of sodium channels: those of the SCN1A and SCN2A sodium channels. In addition, the present invention enables, for the first time, the validation of these three alpha subunits genes as loci, which when mutated, can lead to idiopathic generalized epilepsy (IGE).

At page 7 of the Action, the Examiner asserts that “the predictable correlation between *in vitro* data...and *in vivo* pharmaceutical effects in patients with IGE has not been established”. In view of advancing the prosecution, the Applicant has amended claim 34 to remove the recitation linking the compound to epilepsy. Applicant wishes to state that this deletion should not be construed as an admission that claim 34 as previously drafted was not enabled. Applicant reserves the right to prosecute this deleted subject matter in further applications.

It should also be noted that claim 34 now relates to SCN3A mutants, and that according to the Examiner, the “art does not teach any sodium channel (such as various mutants of SCN3A) can be used in a screening assay.”

At the bottom of page 9 of the Action, relating to the enablement rejection, the Examiner states that “while being enabling for assaying sodium channels using protein with full length SCN3A protein of SEQ ID NO: 67 or the ion channel encoded by the nucleic acid of SEQ ID

NO:65, as well as a method of selecting a compound using in vitro cell based ion channel assay, does not reasonably provide enablement for using any other protein fragments of SEQ ID NO:67 or variants of proteins..., as well as any assays for selecting a compound that can be used for treating IGE.” In view of the above-mentioned amendments to the claims, it is respectfully submitted that this rejection has been overcome. And thus, as stated by the Examiner above, the claims now on file should be enabled.

In view of the amendments to the claims and of the above and foregoing arguments, the Applicant respectfully requests that the Examiner withdraw her rejection of claims 34, 36-41, 43 and 44 under 35 U.S.C. § 112, first paragraph, for failing to comply with the written description and enablement requirements.

III. New Rejections Under 35 U.S.C. §112, First Paragraph

The Examiner rejects claims 14, 34, 36-41, 43 and 44 for failing to comply with the written description requirement.

The Examiner alleges that the recitation of “95% identity” in **claim 14** does not find support in the disclosure. The Examiner also alleges that the specification does not provide support for the claimed “human SCN3A protein associated with idiopathic generalized epilepsy” recited in **claim 34**. These rejections have been rendered moot by the removal of these objected recitations.

IV. New Rejection Under 35 U.S.C. §112; Second Paragraph

The Examiner has rejected claim 36 for using the recitation “the test compound”. In view of the amendment of claim 36 which adds “at least one”, this rejection should have been overcome.

V. Rejections under 35 U.S.C. §102

The Examiner maintains the rejection of claims 14, 34, 37-40, 43 and 44 as being anticipated by Clare *et al.* The Examiner states “although the reference does not explicitly teach the specific nucleic acid sequence (as recited in claim 14: SEQ ID NO:65) or the protein sequence (as recited in claims 14 and 44: SEQ ID NO:67) of the SCN3A (or type III) sodium channel, the specific nucleic acid and amino acid sequences are inherent properties of the human type III (SCN3A) sodium channel.” On page 18, the Examiner further states “the human type III ion channel alpha subunit (or SCN3A) of the reference appears to have the same nucleic acid or amino acid sequences as the ones represented by SEQ ID NOS: 65 and 67 of the instant application, **because the gene and protein sequences for type III ion channel alpha subunit would not be different regardless what the gene or protein is named**”. [emphasis added]

Concerning Applicant’s statement that same does not necessarily agree with the Examiner’s allegations that Clare *et al.* is a meeting paper that should be considered a printed article dating back to May 1998, the Applicant respectfully submits that one cannot determine what was presented in this poster and hence, that the 1998 date should not be construed as a printed publication without any indication of what was disclosed therein.

The Examiner refers to the Applicant’s previous response citing *In re Oelrich* and *Ex parte Cyba*, and rebuts our previous argument that in order to anticipate by inherency, the inherency must be certain, by admitting that “while the cases cited do state that what is asserted to be inherent must necessarily be present, several other cases as well as MPEP § 2112, support the Examiner’s contention that the burden is on applicant to distinguish what is now claimed from the product disclosed in the prior art.” The Examiner then refers to *In re Best* citing for example that “where as here, the claimed and prior art products are identical or substantially identical.”

[emphasis added] ... "The PTO can require an applicant to prove that the prior art products do not necessarily or inherently possess the characteristics of his claimed product."

According to the MPEP, paragraph 2112 in § III, it is stated:

Where applicant claims a composition in terms of a function, property or characteristic and the composition of the prior art is the same as that of the claim but the function is not explicitly disclosed by the reference, the examiner may make a rejection under both 35 U.S.C. 102 and 103... This same rationale should also apply to product, apparatus and process claims claimed in terms of function, property or characteristic.

It should be clear that in the present case the sequence claimed is not claimed in terms of a function or property or characteristic, but in terms of structure. In paragraph IV of the same section of the MPEP entitled "EXAMINER MUST PROVIDE RATIONALE OR EVIDENCE TENDING TO SHOW INHERENCY," states:

The fact that a certain result or characteristic may occur or be present in the prior art is not sufficient to establish the inherency of that result or characteristic. *In re Rijckaert*, 9 F.3d 1531, 1534, 28 USPQ2d 1955, 1957 (Fed. Cir. 1993). [emphasis from MPEP]

In re Oelrich, 666 F.2d 578, 581-82, 212 USPQ 323, 326 (CCPA 1981). To establish inherency, the extrinsic evidence 'must make clear that the missing descriptive matter is necessarily present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill. Inherency, however, may not be established by probabilities or possibilities. The mere fact that a certain thing may result from a given set of circumstances is not sufficient. [emphasis added]

Clearly therefore, the certainty of the allegation as opposed to the possibility thereof is essential, according to case law and the MPEP, for the Examiner to establish inherency. Still in MPEP 2112, at IV,

Also, "[a]n invitation to investigate is not an inherent disclosure" where a prior art reference "discloses no more than a broad genus of potential applications of its discoveries." *Metabolite Labs., Inc. v. Lab. Corp. of Am. Holdings*, 370 F.3d 1354, 1367, 71 USPQ2d 1081, 1091 (Fed. Cir. 2004) (explaining that "[a] prior art reference that discloses a genus still does not

inherently disclose all species within that broad category" but must be examined to see if a disclosure of the claimed species has been made or whether the prior art reference merely invites further experimentation to find the species.

Applicant respectfully submits that the teachings of Clare, are interpretable as a genus sequence, since the claimed nucleic acid sequence of the present invention being of 9112 nucleotides (which the Examiner agrees is not disclosed in Clare) the selection of the claimed sequence SEQ ID NO:65 comes with a probability of $1/(4)^{9112}$. One should thus recognize that the name of the alpha subunit of the SCN3A sodium channel as Type III sodium channel, is a genus comprising a large number of species **one of which is selected** and defined by the present invention as SEQ ID NO:65 (and SEQ ID NO:67) as well as full-length derivatives thereof that would hybridize under stringent conditions thereto. Furthermore, the same section of MPEP states:

In relying upon the theory of inherency, the examiner must provide a basis in fact and/or technical reasoning to reasonably support the determination that the allegedly inherent characteristic necessarily flows from the teachings of the applied prior art. Ex parte Levy, 17 USPQ2d 1461, 1464 (Bd. Pat. App. & Inter. 1990) [emphasis from MPEP]".

Finally, according to section V of MPEP it is only "ONCE A REFERENCE TEACHING PRODUCT APPEARING TO BE SUBSTANTIALLY IDENTICAL IS MADE THE BASIS OF A REJECTION, AND THE EXAMINER PRESENTS EVIDENCE OR REASONING TENDING TO SHOW INHERENCY, THAT THE BURDEN SHIFTS TO THE APPLICANT TO SHOW AN UNOBTAINABLE DIFFERENCE." Applicant respectfully submits that the Examiner has not shown that the claimed sequence of the present invention is substantially identical to that of Clare, based on the fact that it does not teach the sequence, and that it could

not teach a skilled artisan that the name of the gene taught by Clare, amounts to the sequence which is claimed herein.

Applicant respectfully submits that with such a complex gene and the different inconsistencies described in Clare (different sizes of mRNAs, differential splicing, “extreme” unstability of the clones...[see below]), the “**appearance**” that the claimed alpha subunit of SCN3A is the same as that described in Clare cannot be ascertained until further undue experimentation has been carried-out and the task of sequencing this large gene has been completed.

Again, the Applicant respectfully submits that there cannot be an appearance that the two products are substantially identical as discussed above and in the previous response:

- which sequence was subcloned by Clare? Was it the 9.5 kb or the 7.5 kb sequence?

It is impossible to know.

- Could it be a 6kb cDNA, an 8kb cDNA, a 10kb cDNA? **No one knows.**
- Is it SCN3A?

In addition, Clare states that its cDNA’s “were extremely unstable.” Their expression required the growth of E-coli strains at lower temperatures and “correcting spontaneous mutations by site-directed mutagenesis” (page 80). Thus, the skilled artisan reading Clare would expect that the cDNA of Clare, whatever it is, is not the same as that found in nature, and if it is the alpha subunit of SCN3A, is not the same as that of the present invention.

The Examiner alleges that the 7.5 kb band which is detected using a probe from the Type III sequence of Clare, is a spliced variant of the 9.5 kb mRNA. The Examiner then states that “whether or not it is such a variant is immaterial as to the identity of the full length cDNA which encodes a functional sodium channel or of the 9.5 kb band.” The Examiner then goes on to say

“the full length cDNA [**based on what evidence is it full length?**], which encodes a functional sodium channel [**based on what evidence is it deemed functional?**] in the 9.5 kb band isolated on the northern blot is indistinguishable from the nucleic acid now claimed” [emphasis added]. Thus, the Examiner arguably concludes based on circumstantial evidence, and despite the 5% difference in the size of the mRNA detected from a probe derived from the cDNA of Clare and the cDNA of the present invention, that Clare

- cloned the 9.5 kb band (this is neither taught nor suggested);
- that the clone is functional (as compared to what? Based on the assay shown in Figure 1? A number of sodium channels could have given the same results. What is the size of the insert of Clare? On which chromosome(s) does it map? Does it contain restriction sites which are common to the cDNA of the present invention?); and
- that the sequence of 9,112 nt disclosed herein, is the same as the non-shown, non-characterized, non-mapped and non-sized sequence of Clare.

Applicant respectfully submits that this circumstantial evidence “is not sufficient” (MPEP 2112, at IV). Applicant further believes that the Examiner merely bases the inherency rejection on the fact that Clare alleges that his clone contains a Type III sodium channel sequence, that whatever is expressed in his vector shows a Na channel activity, and that it hybridizes to a 9.5 kb mRNA expressed in brain tissue (as stated, the size of the cDNA expressed is not taught or suggested). Applicant maintains that the Examiner has not shifted the burden to the Applicant, because the fact that Clare has indeed cloned SCN3A is at best one possibility (it does “not necessarily flow”...as taught in MPEP, above).

Applicant further refers the Examiner to Annex 1 (Raymond *et al.*, J. Biol. Chem., 2004, Vol 279:46234-46241) which shows different tissue expression of a number of alpha subunit

genes of sodium channels, **including SCN3A**. As clearly shown in Figure 1 thereof, SCN3A is expressed in (1) spinal chord [lumbar, cervical and thoracic], (2) liver, (3) kidney cortex, (4) pancreas, (5) lung, and (6) kidney medulla. Actually, all tissues tested in Raymond *et al.*, showed SCN3A expression. In contradistinction, one of the few things taught by Clare is that the probe derived from the so-called Type III sodium channel cDNA does not detect a band in any of the tissues above. In fact, for all the tissues tested by Clare (in Figure 2, thereof), for which no band is detected, Raymond shows expression, except for placenta and perhaps corpus callosum, which were not tested by Raymond. The Examiner is also referred to page 46237 of Raymond which clearly teaches that the tissue expression model used by the authors provides results that are translatable to humans “the cynomolgus macaque data are a more accurate reflection of normal human biology because the tissue RNA samples were obtained from healthy individuals under carefully controlled conditions...There is a high level of conservation of orthologous sodium channel sequences **and expression patterns** among mammals that is likely even higher among primates.” Applicant respectfully submits that the teachings of Clare, of Raymond, the arguments presented here and those previously presented, strongly support the fact that Clare isolated something else than the alpha subunit of SCN3A. If Clare did indeed isolate the alpha subunit thereof, it should be clear to the skilled artisan that it was far from obvious that his cDNA was the same as that of the present invention.

Finally, Clare *et al.*, is also totally silent on the association of SCN3A and IGE. It is thus respectfully submitted that based on the above and foregoing and based on the recitation of the link between SCN3A and IGE, when mutated, that the inherency rejection in view of Clare must be withdrawn. A request to that effect is earnestly solicited.

VI. Rejections Under 35 U.S.C. §103

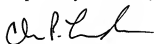
The Examiner has rejected claims 14, 34, 37-40, 43 and 44 under 35 U.S.C. § 103(a) as being rendered obvious by Clare *et al.* in view of Hall.

Applicant respectfully submits that as argued earlier, Clare *et al.*, fails to anticipate or render obvious the methods using the alpha subunit of SCN3A as claimed. The Examiner is referred to the Applicant's arguments above on the lack of inherency of Clare. In view of the fact that (1) Clare does not teach or suggest the SCN3A sequence claimed and the fact that when mutated it can lead to IGE, and (2) that Hall merely provides assays for identifying ion channel modulators using a cation channel of *Drosophila melanogaster*, the defects of Clare are not corrected by Hall, and hence, the combination of Clare and Hall does not render the instant claims obvious. The Applicant therefore respectfully requests that the obviousness rejection be withdrawn.

CONCLUSION

In view of the above, it is submitted that the claims are in condition for allowance. Reconsideration and withdrawal of all outstanding rejections are respectfully requested. Allowance of the claims at an early date is solicited. If any points remain that can be resolved by telephone, the Examiner is invited to contact the undersigned at the telephone number shown below.

Respectfully submitted,



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Annex 1

Expression of Alternatively Spliced Sodium Channel α -Subunit Genes

UNIQUE SPLICING PATTERNS ARE OBSERVED IN DORSAL ROOT GANGLIA*

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Molecular medicine requires the precise definition of drug targets, and tools are now in place to provide genome-wide information on the expression and alternative splicing patterns of any known gene. DNA microarrays were used to monitor transcript levels of the nine well-characterized α -subunit sodium channel genes across a broad range of tissues from cynomolgus monkey, a non-human primate model. Alternative splicing of human transcripts for a subset of the genes that are expressed in dorsal root ganglia, SCN8A (Na_v1.6), SCN9A (Na_v1.7), and SCN11A (Na_v1.9) was characterized in detail. Genomic sequence analysis among gene family paralogs and between cross-species orthologs suggested specific alternative splicing events within transcripts of these genes, all of which were experimentally confirmed in human tissues. Quantitative PCR revealed that certain alternative splice events are uniquely expressed in dorsal root ganglia. In addition to characterization of human transcripts, alternatively spliced sodium channel transcripts were monitored in a rat model for neuropathic pain. Consistent down-regulation of all transcripts was observed, as well as significant changes in the splicing patterns of SCN8A and SCN9A.

Alternative splicing of primary gene transcripts provides a mechanism to generate functionally distinct protein isoforms from a single gene. For the development of safe and efficacious therapeutic compounds, it is necessary to identify the repertoire of proteins that can arise from a gene targeted for therapeutic intervention and determine their tissue distribution within the body. The completion of several mammalian genome sequences, coupled with rich resources provided by extensive expressed sequence tag (EST) and cDNA sequencing, present opportunities for computational prediction of alternative splicing (1–4). The UCSC genome browser (genome.ucsc.edu), which displays overlapping tracks of mRNAs, ESTs, and comparative genomic conservation, can also facilitate the identification of potential alternative splice events. DNA microarrays that monitor exon-exon junctions directly across a broad range of transcripts provide an additional resource to detect alterna-

tive splicing on a genome-wide scale (5, 6). These combined computational and experimental approaches, coupled with traditional laboratory validation, provide a wealth of information about alternative splicing and tissue-specific expression, which are essential to define a drug target at the molecular level.

Sodium channels are multisubunit protein complexes that play a pivotal role in the propagation of action potentials along neurons (7, 8). The α -subunit genes encode the primary channel-forming pores within the cell membrane that allow ion-specific translocation. The α -subunit gene family contains nine paralogs (and one additional sodium channel-like gene, Na_v2) that are highly conserved across vertebrate species (9). The channel protein structure includes four highly similar clusters of transmembrane helices that are connected by intracellular loops. A similar structure is found in calcium and potassium channels, indicating that this ion channel superfamily arose from a single primordial ion channel gene (8, 10). Voltage-gated sodium channels perform a broad spectrum of functions within vertebrate cells, as is evident from the large number of paralogous genes and their tissue-selective expression patterns. For a particular sodium channel gene, subtle differences in channel properties can be attributed to alternative splicing, post-translational modification, changes in the expression of ancillary β -subunits, and mutation (7, 8). Importantly, alternative splicing of transcripts derived from a common gene has been shown to generate biochemically and pharmacologically distinct sodium channel isoforms (11, 12).

We chose to focus our attention on sodium channels expressed in dorsal root ganglia (DRG),¹ peripheral nervous system (PNS) structures found just outside the spinal column that play key roles in sensory transmission from the periphery to the brain. Channels expressed in DRG are known to play key roles in nociception (7, 8, 13). Modulation of DRG sodium channel activity may provide relief from neuropathic pain, a medical condition that is not well addressed by current medicinal therapies (13). Our goal was to catalog and quantify alternative splicing events that occur in SCN8A (encoding Nav1.6, PN4), SCN9A (encoding Nav 1.7, PN1), and SCN11A (encoding Nav 1.9, PN5). As an example, previous research had shown alternative splicing of SCN8A transcripts (14, 15). In rat DRG, alternative splicing extends the reading frame of exon 11, resulting in a channel that has altered kinetics of inactivation and reactivation relative to the non-extended isoform (12). Developmentally regulated alternative splicing of SCN8A coding exon 18 in mouse and human results in a transcript that encodes a truncated, nonfunctional channel that appears in

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY682082, AY682081, AY682083, AY682085, AY682084, AY682086, and AY682084.

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¹ The abbreviations used are: DRG, dorsal root ganglia; PNS, peripheral nervous system; nt, nucleotides; RT, reverse transcriptase; CNS, central nervous system.

TABLE I
PCR primers used for RT-PCR

Primer name	5' to 3' sequence	Purpose
SCN8.5F	CAAGAGGTTTCCTGATAGTGGCTTAC	Amplify human SCN8A exon 5 to exon 13
SCN8.13R	CATTATACCTTGATCTCTCTCTCCGC	Amplify human SCN8A exon 5 to exon 12
SCN9.4F	TGTGTAGAGAACTACCTTTCTCTCTG	Amplify human SCN9A exon 4 to exon 13
SCN9.12R	GGAGTAGAGAACTACAGCCCTTTCTT	Amplify human SCN9A exon 4 to exon 12
SCN11.15F	GAATCCTGAGATTTCTCTGGGAGATA	Amplify human SCN11A exon 15 to exon 19
SCN11.15R	CAATTCGAGGCTACCATTTTAGTA	Amplify human SCN11A exon 15 to exon 19

TABLE II
GenBank™ accession numbers for deposited partial mRNA sequences

DNA sequence	GenBank™ accession number
Human SCN8A - exon 6N to exon 12EXT	AY682082
Human SCN8A - exon 6A to exon 12BS	AY682081
Human SCN8A - exon 6A to exon 12EXT	AY682083
Human SCN9A - exon 5N to exon 11EXT	AY682086
Human SCN9A - exon 5A to exon 11BS	AY682084
Human SCN9A - exon 5A to exon 11EXT	AY682086
Human SCN11A - exon 15 skip	AY686224

TABLE III
TaqMan® primer probe sets used to quantify alternative splicing

TaqMan® assay	5'-3' Forward primer sequence 5'-3' Reverse primer sequence 5'-3' Probe sequence
Human SCN8A exon 6N	ACAGAGTTTGTAAACCTAGGCAATGT GCCTGGGATACCGAAATAGTTT FAM ^a -CTGAAAGCTGTAGAGCTG-NFQ ^b CTGGCTCTGGCAATGCT
Human SCN8A exon 6A	AGAGATAGTTTCTAAAGCTCGGAGAAC FAM-CAGCGCTGAGACAT-NFQ CCCGGCTCCACATC
Human SCN8A exon 12wt	CCAGGGCTTCTCTTCTTAATTTCCA FAM-TCTGCTGAGAGGCTAC-NFQ CTCCGAGGAGTGAATATGATAGG
Human SCN8A exon 12ext	CCAGGGCTTCTCTTCTTAATTTCCA FAM-CCGATGACAGTGTCTACAC-NFQ CTGGATTTTGTGCTCATTTGTTTGTG
Human SCN9A exon 5N	CAAGGCCCTACATTTGCTTCAG FAM-TTTCAGCTCTTCTGCACTT-NFQ CTGGATTTTGTGCTCATTTGTTTGTG
Human SCN9A exon 5A	CAAGGCCCTACATTTGCTTCAG VIC ^c -AGCATTTGAGACATTCAG-NFQ TCCCAATGAGACAGCTTCTG
Human SCN9A exon 11wt	GGAGATAGGACATACAGGCTTTT FAM-CCAGAGGACACGACCA-NFQ AGCTTCTGCAAGGTTGATATAGA
Human SCN9A exon 11ext	GGAGATAGGACATACAGGCTTTT FAM-ACAGGCGACAGACCA-NFQ TCACACACCTGAGGCTGAG
Human SCN11A wild-type	GTGGCTTTCTTGTCTCTGAT FAM-ACAGGCGCTATGAGCTCC-NFQ ACAGGCTATACAGACCT
Human SCN11A exon 16 skip	CTCAAGATAGTGTCTGACATCTG FAM-CTCGAGATACAGAGTCT-NFQ ACAGAGTTTGTAACTAGGCAATGT
Rat SCN8A exon 5N	GGCTGGGATACCGAAATAGTTT FAM-CTGAAAGCTGTAGAGCTG-NFQ CAGGTTTCTCCGAGTTTGA
Rat SCN8A exon 5A	CCGCCAGAGTTTCTTCTGAG FAM-CTGGATTTCTGAGGCTG-NFQ CGCCCGCTCACAT
Rat SCN8A exon 11wt	GCAGGCTTCTTCTTCTTAATTTCCA FAM-CTGCTGAGGACAG-NFQ CTCTCTGCTGAGGAGAAATAGATA
Rat SCN8A exon 11ext	CCAGGGCTTCTTCTTCTTAATTTCCA FAM-CTGCTGAGGAGAAATAGATA TTTGTCTATTTTCTGATTTTACAGAA
Rat SCN9A exon 5N	CCTGGATATACAGAAATAGTTTCAAAAG FAM-TTCAGGCTGAGGCTGAGCTT-NFQ ACATTCAGAGTTCTCCGAGATTTG
Rat SCN9A exon 5A	CCGCCAGAGTTTCTTCTGAG FAM-CCGAGATTTCTGAGGCTG-NFQ GCTCCCAATGAGACCT
Rat SCN9A exon 11wt	GACAAGATAGGACATGAGAGGCTTT FAM-CCAGAGGCGACAGTAA-NFQ GACAGCTTCTCCAGAGGTAATAA
Rat SCN9A exon 11ext	GACAAGATAGGACATGAGAGGCTTT FAM-ACAGGCGGACAGTAA-NFQ CTTACAGCTTCTGAGGCTG
Rat SCN11A wild-type	GGCTAGTGAAGCTCTGCTG FAM-CCCGGCTGCACTT-NFQ

^a FAM, FAM fluorophore.^b NFQ, non-fluorescent quencher.^c VIC, VIC fluorophore.

fetal tissue but vanishes just after birth (14). Finally, a splicing event involving a mutually exclusive alternative exon 6 was inferred from human genomic sequence (15). A recent report of DRG-selective expression of an alternatively spliced, functionally distinct variant of the voltage-gated calcium channel *CACNA1B* (Cav2.2) suggests that DRG-specific alternative splicing may generate a unique constellation of ion channels within this physiologically important PNS subregion (16). In this study, we were able to detect both known and novel sodium channel transcripts in DRG that arise by alternative splicing. Quantitative PCR was used to assess the expression patterns of these alternatively spliced isoforms, and we found that alternative splicing of sodium channel mRNAs was most pronounced in DRG. These data indicate that effective treatment of neuropathic pain by antagonism of sodium channels must account for multiple channel isoforms.

MATERIALS AND METHODS

Sodium Channel Expression Compendium (Body Atlas)—Gene expression profiling of cDNA libraries was performed on in-house synthesized oligonucleotide microarrays designed to monitor ~47,000 human transcripts. This two-array set (Hu5K) is an updated version of human microarrays described previously (17). Probes for the nine voltage-gated sodium channel transcripts reported here were present on these arrays. Additional results from these experiments will be described in future publications. Microarray probe sequences were designed to hybridize near the transcript 3'-end. Messenger RNA amplification and hybridization conditions were performed as described previously (17). Individual samples were labeled with either fluorescent Cy3 or Cy5 dye and hybridized to a human microarray in replicate against a mass-balanced control pool of 220 individual tissue RNA samples. Each experiment was repeated with the Cy3 and Cy5 dyes reversed (a dye swap). Microarrays were purchased from Agilent Technologies (Palo Alto, CA). The housing, necropsy, and extraction of RNA from organs and tissues of all animals used in the monkey expression experiments was performed by MPI Research Inc. (Mattawan, MI), with the sole exception of RNA extraction from bone samples. Four cynomolgus (*Macaca fascicularis*) monkeys, 2 male and 2 female, were the source of the organs and tissues. All animals were matched for age, weight, and diet (Lab Diet® Certified Primate Diet 5048, PMI Nutrition International, Inc.) available *ad libitum*. Monkeys were fasted for 16 h pre-euthanasia and sedated with ketamine, followed by overdose of sodium pentobarbital solution and exsanguination. All samples were harvested, trimmed, and snap frozen in liquid nitrogen within 30 min with the majority of samples frozen within 15 min.

Each sample was hybridized to 8 microarrays: 4 individuals and 2 microarrays for the dye-swap. For each gene, a \log_{10} ratio of individual sample to pool was generated by combining dye-swap microarray pairs. These 4 values, one for each individual, were then averaged in an error-weighted fashion (18) to produce a \log_{10} error-weighted average

(tissue-to-pool) ratio for each gene. Error bars estimate the one standard deviation of this average because the modeled errors calculated for each sample and the replicate error (18). Finally, for each gene, we

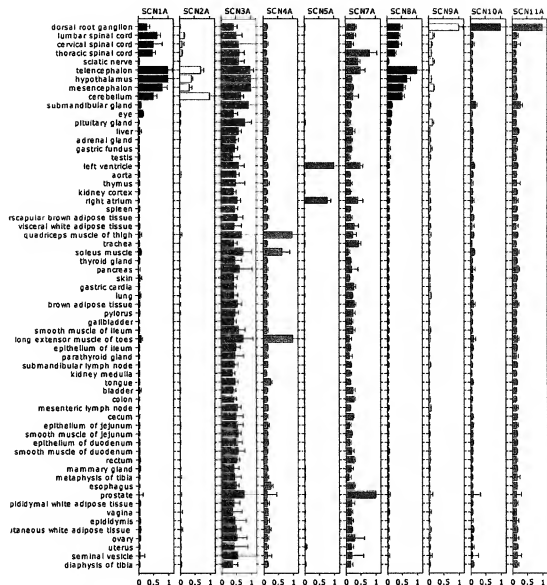


FIG. 1. Sodium channel body atlas from cynomolgus macaque monkey. Microarray measurements were made of sodium channel α -subunit transcripts across multiple tissue-derived RNA samples. For each gene, fluorescence intensity values were measured for labeled cRNA hybridized to 3'-positioned probe sequences. The tissue sample that generated the highest intensity value was assigned a value of 1, and all remaining samples were normalized on a linear scale. The tissues were sorted by SCN8A expression level, first by PNS, then CNS, and then all remaining tissues. Error bars estimate 1 S.D. of average measurements and are described in more detail under "Materials and Methods."

transformed the log ratios to ratios and normalized linearly by scaling the largest tissue ratio to 1.

RT-PCR and Quantitative Real Time PCR (TaqMan®)—Reverse transcription-polymerase chain reaction (RT-PCR) amplification from tissue-specific mRNA or total RNA was performed as described previously (5). The oligonucleotides used in this study (Table I) were obtained from Qiagen (Valencia, CA). Amplifications were subcloned into pCR2.1 using a TOPO-TA cloning kit (Invitrogen). Sequencing was performed by a commercial vendor (Lark Technologies Inc., Houston, TX). The sequences of all isoforms described in this study were deposited into GenBank™ (Table II).

TaqMan® is a registered trademark of Roche Applied Science. TaqMan® primer probe reagents were obtained through the Applied Biosystems Assays-by-Design custom assay service (Foster City, CA). The primer-probe sets used in this study are shown in Table III. Probe sequences were designed to straddle the unique splice junctions characteristic of each alternative splice form. TaqMan® assays were performed on an ABI 7900 real time PCR instrument in 10- μ l assays that were run in triplicate in a 384-well format optical PCR plate. The assays were calibrated with isoform-specific RT-PCR clones using the

standard curve method.² Standard curves generated from plasmid clones were linear across at least six orders of magnitude, and all reported values derived for total tissue RNA fell within the range of these standard curves.

Total RNA from human tissue was obtained from Clontech. Total rat dorsal root ganglia RNA from control and treated animals from a spinal nerve ligation neuropathic pain model was obtained as a gift from Dr. Hao Wang and colleagues (Merck Research Labs, West Point, PA). All of the handling of the animals and testing was performed in accordance with the policies and recommendations of the International Association for the Study of Pain (19) and received approval from the Institutional Animal Care and Use Committee of MRL, West Point, PA. The experimental treatment of the animals was exactly as described in Ref. 20. RNA was converted to cDNA for TaqMan® measurements using a commercially available kit from Applied Biosystems. All assays were normalized on a tissue-to-tissue basis by adding a constant amount of

² Essentials of Real Time PCR, www.appliedbiosystems.com/support/tutorials/pdf/essentials_of_real_time_pcr.pdf

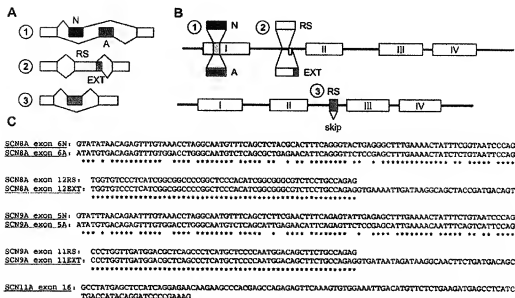


Fig. 2. Alternative splicing of SCN8A, SCN9A, and SCN11A transcripts. **A**, neonatal (N) and adult (A) alternative splices involve the use of mutually exclusive alternative exons. The RefSeq (RS; human SCN8A [NM_014191.1], human SCN9A [NM_002977.1]), and extended (EXT) coding exon 11 sequences are generated by the use of alternative splice donor sites. The SCN11A variant was generated by exon skipping (3). **B**, sodium channel transcripts are represented as a line, with the encoded pore-forming transmembrane domains shown as numbered rectangles. SCN8A and SCN9A are represented by the top gene structure. Both genes undergo analogous splicing events. SCN11A is shown as the bottom transcript with the position of the exon-skipping event denoted. **C**, nucleotide sequences of alternative exons, exon extensions, and the skipped SCN11A exon.

input total RNA into the RT reaction. We chose this normalization method because we were unable to identify a single housekeeping gene that yielded satisfactory normalization data. Isoform levels within tissue RNAs were measured in triplicate on separate occasions, and the results were highly reproducible. A representative data set is shown in each case.

Quantitative PCR values were calculated by assuming that the sum of splicing events at a given site was equal to unity (e.g., SCN8A [exon 6N] + [exon 6A] = 1). This was applied to the most abundant measurement in each data set (SCN8A-adult brain; SCN9A exon 5-DRG; SCN9A exon 11-fetal brain) where the sum of the isoform measurements was adjusted to a value of 100%. All other measurements in the data set were normalized to these maximum values. Error bars are 1 S.D. of the average of triplicate measurements.

RESULTS

Body Atlas Expression Patterns of Sodium Channels—DNA microarrays afford the opportunity for genome-wide monitoring of transcription within any RNA sample. When RNA samples from diverse tissues throughout the body are hybridized, it becomes possible to assemble a transcriptional compendium or Body Atlas (5, 21, 22). This resource is essentially a semiquantitative, whole-genome Northern blot for all transcripts with corresponding probes on the array. The Body Atlas of the nine voltage-gated sodium channel paralogs across a broad range of tissues was obtained for cynomolgus macaque monkey transcripts profiled on human microarrays (Fig. 1). While entirely consistent with previous studies (7–9), these data are unique in that they provide a comprehensive overview of the entire gene family in a wide range of tissues. We present the data for monkey because our data for human were incomplete. Moreover, we believe the cynomolgus macaque data are a more accurate reflection of normal human biology because tissue RNA samples were obtained from healthy individuals under carefully controlled conditions. Microarrays using human sequences have routinely and successfully been used to profile non-human primate samples to study gene expression, including the use of samples from cynomolgus monkey (23). There is a high level of conservation of orthologous sodium channel

sequences and expression patterns among mammals that is likely even higher among primates. On average, one would expect zero to only a few mismatched bases between monkey and human per 60-mer probe sequence. Of the 10 sodium channel probes that could be mapped to both human and chimpanzee genomic sequences, seven were perfect matches in both species. Finally, although sequence differences between human and cynomolgus monkey transcripts may affect individual probe intensities, they are unlikely to influence intensity ratios (between sample and pool), which are used here. Examination of the Body Atlas data reveals that SCN4A (Na_v1.4) and SCN5A (Na_v1.5) exhibit strikingly selective expression in striated muscle and heart, respectively. SCN3A (Na_v1.3) appears to be transcribed in numerous tissues. Channels SCN1A (Na_v1.1), SCN2A (Na_v1.2) and SCN8A (Na_v1.6) appear to be abundantly expressed in both PNS and CNS tissues. In contrast, SCN9A (Na_v1.7), SCN10A (Na_v1.8) and SCN11A (Na_v1.9) expression is strikingly selective to DRG, with only minor expression levels detected elsewhere in the body.

Detection of Alternative Splicing in SCN8A, SCN9A, and SCN11A—Within the vertebrate sodium channel paralog family, parsimonious clustering by protein sequence indicates that SCN8A and SCN9A occupy one branch of the sodium channel family tree that also includes SCN1A, SCN2A, and SCN3A (8, 9). As mentioned, within SCN8A, two alternative splicing events with the potential to produce functional sodium channels have been described. The first involves the potential use of mutually exclusive, alternative exon 6 (coding exon 5) sequences that encode parts of transmembrane segments S3 and S4 within domain I (Fig. 2 and Refs. 14 and 15). The 92 nucleotide (nt) alternative exons, which are found in human, mouse, and rat genomic sequence and known to be used in other sodium channel family members, code for nearly identical amino acid sequences that differ at only two positions. The second, described in mouse and rat, involves the use of alternative 5'-splice donor sites in exon 12 (coding exon 11), which

encodes a portion of the cytoplasmic loop between domains I and II (Fig. 2 and Ref. 12). The resulting channels differ by 11 amino acid residues, and these isoforms exhibit distinct electrophysiological properties (Table I and Ref. 12). Given conservation of the respective genomic sequences, we hypothesized that these alternative splice events might be expressed in human tissues. Gene-specific amplification primers for SCN8A were used to generate RT-PCR products from human DRG, and sequencing confirmed usage of both mutually exclusive exon 6 cassettes and alternative splice donor sites in exon 12 (Fig. 2). Inspection of genomic sequence of human, mouse and rat SCN9A suggested it has a gene structure similar to SCN8A. Specifically, the genome sequences from all three species encode potentially mutually exclusive exon 5 sequences, and evidence for alternative splicing of the rabbit paralogue was deduced from cDNA sequences (24). Similarly, conservation of exon 11 alternative splice donor sites was found in human, mouse and rat genomic sequences, and evidence for alternative splicing was suggested by comparison of human, rat, and rabbit cDNA sequences (12). Confirmation that both of alternative splice events occur in human SCN9A was obtained by sequencing of RT-PCR products amplified from human DRG (Fig. 2).

Cross-species comparative studies using a combined paralog/ortholog approach also revealed a novel, alternatively spliced isoform of SCN11A. Sodium channel genes SCN5A, SCN10A, and SCN11A share a similar gene structure (25). Murine SCN5A is alternatively spliced in heart tissues, with one transcript that is missing the RefSeq (NM_021544.1) exon 17 (26). The same splicing event is observed in rat transcripts of SCN5A (GenBank™ AF353637). This 159-nt exon codes for 53 amino acids situated in the cytoplasmic loop region between domains II and III. Electrophysiology measurements suggest that the Na_v1.5 channels encoded by these isoforms are functionally similar (26). The human SCN11A gene encodes an analogous 114 nt, 38 amino acid exon in the same cytoplasmic loop-encoding region of the transcript. Moreover, this loop region has only 48% protein sequence identity between human and mouse, whereas the overall channel identity is 72%. Therefore, by analogy to murine SCN5A, this splice variant of SCN11A may encode a functional sodium channel. RT-PCR across this region of human SCN11A using DRG total RNA gave predominantly the expected exon 16⁺ amplicon, however a clone containing a smaller amplicon was isolated, and sequencing revealed it encoded a splice variant lacking exon 16.

Quantitation of Alternatively Spliced Isoforms—Real-time PCR, wherein exon-specific primers flank a splice junction-specific, fluorescently labeled probe (commonly referred to as a TaqMan® assay) was used to quantify alternative splicing events. Using custom assays, we monitored the ratios of all of the alternative splicing events shown in Fig. 2 across two PNS (DRG and spinal cord) and two CNS (fetal and adult whole brain) tissue RNA samples (Fig. 3).

Several interesting conclusions can be drawn from these data. First, the tissue-specific differences in the overall expression levels of each channel transcript closely mirror the Body Atlas array measurements shown in Fig. 1. Second, almost all alternative splicing events are well represented, especially in DRG. This is particularly true of SCN9A, where we observed roughly equal abundance of both exon 5 and exon 11 alternative splice variants. The expression levels and splicing patterns observed in spinal cord were distinct from DRG, especially for SCN9A. Third, despite the relatively high abundance of certain splicing isoforms (e.g. SCN8A transcripts that include exon 6A), many of these alternatively spliced transcripts were not

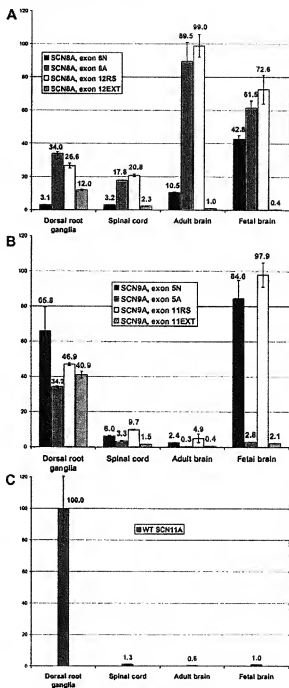


Fig. 3. Quantitative TaqMan® measurements of alternative splicing. Junction-specific TaqMan® assays were calibrated against control plasmids by the standard curve method, and validated with control reactions against alternative junction sequences. The data were normalized to the highest measurement, which were assigned an arbitrary value of 100. Error bars are the standard deviations of triplicate measurements. Abbreviations are the same as Fig. 2. A, expression of alternative splice forms of SCN8A. B, expression of alternative splice forms of SCN9A. C, expression of SCN11A. The exon 16 skip variant was below the limits of reliable detection.

found in GenBank™. Finally, we were unable to reliably detect the SCN11A Δexon 16 splice variant, suggesting it is expressed at very low levels in human DRG (data not shown).

Alternative Splicing Events Appear to Be Unlinked—In DRG, SCN8A, and SCN9A transcripts undergo frequent alternative splicing at two sites (exon 6N versus 6A and exon 12RS versus

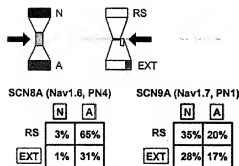


Fig. 4. Alternative splicing events in SCN8A and SCN9A transcripts appear to be uncoupled. PCR primers (arrows) were used to generate independent clones of SCN8A (287 clones) or SCN9A (92 clones) from DRG cDNA that span both alternative splice sites. TaqMan® assays were used to determine the alternative splicing events within each clone. The frequency of each clone is represented as a percentage.

exon 12EXT for SCN8A; exon 5N versus exon 5A and exon 11RS versus exon 11EXT for SCN9A). It is possible that these alternative splicing events are linked, e.g. SCN9A transcripts with exon 5N generally possess exon 11EXT. Conversely, alternative splicing at one site may not influence splicing at a distal site, in which case there should be a stochastic distribution of splicing-generated isoforms. To test this, we amplified cDNA prepared from DRG with PCR primers that span both alternative splice sites for SCN8A and SCN9A (Fig. 4). Individual amplicon clones were then screened at both alternative splice positions to determine the splicing pattern of each clone. We found all possible combinations of splice events, and the distribution of splicing events within the overall set of clones suggested that alternative splicing events were regulated independently with respect to one another. The implication of this finding is that DRG is populated with at least four distinct alternatively spliced isoforms of both SCN8A and SCN9A.

Sodium Channel Expression and Alternative Splicing in a Rat Neuropathic Pain Model—Nerve damage in the periphery can result in chronic neuropathic pain. Effective treatments for this condition may result from a more complete understanding of the biological changes that accompany nerve injury. We monitored isoform-specific changes in expression levels that occur in response to spinal nerve ligation in rat, a commonly used model for neuropathic pain (27). Total RNA from ipsilateral DRG of control-treated and nerve-damaged animals was harvested during the period in which maximal allodynia was observed in the injured animals (2 weeks post-injury). In control animals, the splicing pattern of rat SCN8A appeared similar to human SCN8A (Fig. 5A). In contrast, alternative splicing of rat SCN9A transcripts in control DRG was quantitatively different from human (Fig. 5B). In rat, 80% of the SCN9A transcripts include the exon 11 extension variant, whereas in human, this variant appeared to make up 45% of the overall DRG SCN9A transcript. The significance of this species-specific difference is unclear.

As reported previously, down-regulation of the SCN8A, SCN9A, and SCN11A sodium channel transcripts was observed in ipsilateral DRG in response to neuropathic injury (20, 28–30). SCN8A expression is reduced to 17% of control (Fig. 5A), SCN9A to 27% (Fig. 5B), and SCN11A to 2.5% (data not shown). We also observed a significant change in the expression pattern of alternatively spliced isoforms. In Fig. 5C, we show the retention of alternative splice forms, which is simply the post-injury value divided by the control value for each individual splicing event. For SCN8A, selective retention of

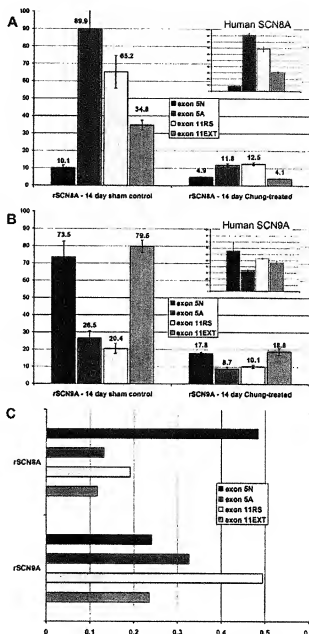


Fig. 5. Quantitative PCR measurements of SCN8A and SCN9A alternative splicing in a rat spinal nerve ligation model. Assays designed against specific rat sequences were used to measure alternative splice variants in DRG total RNA isolated from control and neuropathic animals by the standard curve method. Measurements of exon 5A + exon 5N or exon 11RS + exon 11EXT for rat SCN8A or rat SCN9A in untreated DRG were assigned a value of 100. The corresponding measurements made for treated samples were normalized to these values. A, SCN8A expression and alternative splicing in control and Chung-treated rat DRG. The splicing pattern of human SCN8A in DRG is shown in the inset for comparison. The numbering of the rat SCN8A exons is different from human SCN8A owing to the absence of a 5'-UTR exon in rat RefSeq sequence. B, SCN9A expression and alternative splicing in the rat neuropathic pain model. The human SCN9A splicing pattern from DRG is shown in the inset. C, retention of alternative splice events in response to neuropathic injury. Values were calculated by dividing the treated values by control values for each individual splicing event.

exon 6N was observed. For SCN9A, the exon 11RS-containing transcripts were enriched in relative abundance in response to treatment.

DRG, although this isoform appears to be rare. Further studies will determine if this transcript encodes a functional sodium channel.

Dorsal root ganglia are clearly unique with respect to sodium channel expression and alternative splicing. Specific sodium channel genes are expressed in DRG and almost nowhere else in the body. Moreover, alternative splicing within these transcripts generates a potentially diverse set of sodium channel isoforms. Similar DRG-selective alternative splicing of calcium channel CACNA1B transcripts has been reported recently (16). DRG is composed of heterogeneous cell types that were shown to differ in their expression patterns of CACNA1B transcripts. Similar observations were made with SCN9A transcripts in rat DRG (35). In this study, we have treated DRG as a homogeneous tissue and detected a diverse spectrum of alternative splicing. It will be of interest to determine whether unique transcripts are constrained to specific cell subtypes, and more importantly, which sodium channel isoforms contribute most to neuropathic pain. Such channels would be preferred targets of a future class of channel-specific antagonists. Our investigation of a rat neuropathic pain model revealed selective enrichment of SCN9A mRNAs encoding exon 5N and SCN9A transcripts that included exon 11RS, suggesting these isoforms may selectively contribute to neuropathic pain.

Examination of DRG-specific, voltage-gated sodium channels highlight an important theme with respect to basic biology and pharmaceutical compound development. We found a surprising diversity of alternative splice forms in the highest expressing tissue, DRG, which is also a region of therapeutic focus with respect to neuropathic pain. Our search was by no means exhaustive. We did not examine SCN10A transcripts for alternative splicing and are not certain we have identified all of the highly expressed splicing events that occur in SCN8A, SCN9A, or SCN11A. While our current knowledge of the human transcriptome is a powerful resource, we believe that much remains to be discovered about alternative splicing and that a thorough knowledge of these post-transcriptional events will be critical to the development of more effective and specific therapies in the treatment of disease and the maintenance of health.

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